

Identification of a Major Xylanase from *Aspergillus flavus* as a 14-kD Protein

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Abstract *Aspergillus flavus* K49 secreted at least two xylanase activities when grown on a medium containing larch (wood) xylan as a sole carbon source. Enzyme activity was assayed using an agar medium containing Remazol Brilliant Blue R conjugated oat spelt xylan as substrate. Crude enzyme preparations were inhibited by Hg^{+2} , with an ED_{50} of 17.5 mM and maximum inhibition of 83% at 50 mM. A concentrated sample of *A. flavus* K49 xylanase preparation was subjected to gel filtration chromatography on a P-30 column. A small protein peak coinciding with the major peak of xylanase activity was separated from the other secreted fungal proteins. An additional peak of xylanase activity was observed in fractions containing multiple fungal proteins. Analysis by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of fractions containing the

smaller molecular weight xylanase revealed a major and minor protein band in the vicinity of 14 kD. Analysis of these same fractions by acidic native PAGE revealed a single band. Confirmation of identity for the isolated xylanase was provided by isolation of a protein band from a SDS–PAGE gel, followed by trypsin digestion/analysis by tandem mass spectrometry. Comparison of the peptide library derived from this protein band with sequence data from the *A. oryzae* genomic data base provided a solid match with an endo-1,4- β -xylanase, XlnA. This identification is consistent with a low molecular weight protein associated with the major xylanolytic activity. XlnA may be a highly mobile (diffusible), plant wall hemicellulose degrading factor with significant activity during plant infection.

Keywords *Aspergillus flavus* · Xylanase · Hemicellulose · Hydrolase

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Introduction

Aspergillus flavus is a ubiquitous saprophytic fungus commonly found in tropical and subtropical climes. This organism is also an opportunistic pathogen of a number of oilseed crops and has agronomic significance due to its production of the potent carcinogenic mycotoxin aflatoxin (B_1) [1]. Plant host invasion by *A. flavus* is enabled by production of a

large array of hydrolytic enzymes, both for nutrient capture and penetration of host plant tissues [2]. Since plant reserve materials (free sugars, lipids, polysaccharides, proteins) that are potential fungal substrates are generally encapsulated within protective wall tissues, production of hydrolases capable of aiding wall penetration is a selective advantage to the fungus.

Xyloglucans and xylans comprise the hemicellulose fraction of plant cell walls. They function within the cell wall to help (along with pectins) give proper orientation to the cellulose microfibrils (primary wall) and allow for wall expansion during wall growth [3]. Endoxylanase activities hydrolyze the xylan backbone of these plant polysaccharides in a random fashion at non-modified internal residues, allowing for the production of xylo-oligomers. Thus, this activity provides a major contribution toward plant cell wall maceration.

In addition, xylanases have industrial applications. Xylanolytic activities are used in environmentally safe bleaching of pulps in the pulp and paper industry [4]. They are also used in the baking industry to improve dough quality and in the animal feed industry to increase feed conversion efficiency [4].

A. flavus AF36 secreted a xylanase activity when grown on a medium containing larch xylan as a sole carbon source. Some preliminary work indicated this xylanase activity is highly conserved in *Aspergillus* section *Flavi* [5]. In addition, this activity is thermostable, tolerant of a wide pH range, and is the product of a small protein (≤ 20 kD). Five genes are annotated as xylanases in the *A. flavus* genome [2], but the contributions of each gene in saprophytic or pathogenic modes are unknown. Identification and characterization of xylanases, both *in vivo* and *in vitro*, is necessary to understand the pathology of *A. flavus* infections of maize and other oilseed crops.

Materials and Methods

Biological Materials

Non-toxigenic *Aspergillus flavus* isolate K49 (NRRL 30797, public access NCAUR collection) was isolated by H. Abbas from maize kernel material obtained from field studies [6]. This isolate was maintained on standard potato dextrose agar at 28°C.

Fungal conidial suspensions were constructed with sterile deionized water and contained $1\text{--}2 \times 10^6$ conidia per ml. Larch xylan and Remazol Brilliant Blue R were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Xylanase Production in Liquid Culture

A chemically defined culture medium [7], containing dextrose (2 g/l) and insoluble larch xylan (0.35 g/flask) as a carbon source and sodium nitrate (3 g/l) as a nitrogen source [8], was used as a fungal growth medium. Control cultures contained 0.49 g of dextrose per flask as a sole carbon source. The medium was adjusted to pH 5.5 before heat sterilization. Incubations were conducted in 70 ml of medium in 250-ml flasks. Each incubation flask was seeded with 200 μ l of a conidial suspension. Fungal fermentations were carried out in a shaking incubator (150 rpm) in the dark at 30°C for 6 days. Filtrates of fermentations were used as sources of crude xylanase activity after being filter-sterilized (0.22 μ m filter).

Xylanase Activity Assay

Oat spelt xylan was conjugated with Remazol Brilliant Blue R (RBB) according to a previously published procedure [9]. Activity was observed by means of a semi-quantitative radial diffusion assay in a medium containing 0.05% (w/w) RBB-xylan and 2% agar (9-cm gel plates). Digestion of RBB-xylan produced a circular expanding clear zone surrounded by a thin (1 mm) layer displaying a higher intensity of blue color (RBB-xylan fragments). Wells, 5 mm in diameter, were cut into the gel medium and filled with test solution (50 μ l). Assay plates were incubated at 37°C (dark) for 5–6 h. The diameter of each digestion zone was measured and activity level is reported as area of the RBB-xylan digestion zone (total area less well area).

Inhibitor Studies

Test solutions were prepared with crude *A. flavus* xylanase (sterilized growth medium) in a concentration range of 0–50 mM mercuric chloride. Test solutions were pre-incubated at 25°C for 15 min before introduction to the xylanase assay medium. Inhibition values were calculated in comparison of

test solution digestion zones to positive control values (0 mM Hg⁺²).

Purification of *A. flavus* K49 Xylanase

BioGel P-30 (BioRad Labs, Richmond, CA, USA) gel filtration medium was equilibrated with 0.05 M 2-(N-Morpholino) ethanesulfonic acid (MES), pH 6.2, packed to form a 1.5 by 83-cm column (bed vol. = 147 ml; void vol. = 50 ml), and thoroughly washed with MES buffer. *A. flavus* K49 sterile culture filtrate, 200 ml, was dialyzed exhaustively against deionized water and lyophilized to dryness. This dried preparation was re-solvated in 2.0 ml of 0.05 M MES, pH 6.2, applied to the column, and eluted with MES buffer. After 30 ml of column eluant had passed through, sixty-five 2-ml fractions were collected. Column eluant (continuous flow) was monitored for A_{280nm} and conductivity. Column fractions were assayed for protein concentration (A_{280nm}) and xylanase activity (radial diffusion assay).

Electrophoretic Analysis

Analytical tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed generally according to the method of Schägger and von Jagow [10]. This gel system is designed to yield superior resolution of proteins with molecular weights between 6 and 30 kD. Gels (16.5% T, 3% C; 0.08 × 8 × 7 cm) were run at 150 V (constant) at 25°C. Aliquots from column fractions (MES buffer) were diluted 1:3 with sample buffer containing 50 mM dithiolthreitol (DTT) and heated at 100°C for 5 min. Protein molecular mass standards were obtained from Novex (San Diego, CA, USA). Proteins were detected by a modified silver stain procedure [11].

Analytical native acidic PAGE used for separation of basic proteins has been described [12]. A number of modifications to the cited system were instituted. The separatory gel buffer (lower gel) consisted of 250 mM potassium acetate, pH 4.3, while the stacking gel buffer (upper gel) was 500 mM potassium acetate, pH 6.3. The sample buffer was 94 mM glycine, adjusted to pH 4.3 with acetic acid and also contained 10% (v/v) glycerol. The anodic reservoir buffer consisted of 75 mM glycine/acetate, pH 4.0, and the cathodic reservoir buffer was 75 mM

potassium acetate, pH 4.3. Gel polymerization required increased concentrations of ammonium persulfate and TEMED (three- to fourfold greater than with basic native PAGE), due to reduced polymerization efficiency in acid conditions. Gels (15% T, 3% C; 0.08 × 8 × 7 cm) were run at 150 V (constant). Proteins were detected with the silver stain procedure cited previously. Aliquots from test column fractions were diluted 1:4 with gel sample buffer. Gel samples received no heat treatment prior to electrophoresis.

Xylanase Identification Verification

Denatured SDS–PAGE was performed generally according to the method of Laemmli [13]. Gels (12% T, 3% C; 0.08 × 8 × 7 cm) were run at 90 V (constant) when samples were in the stacking gel; 125 V (constant) in the resolution gel. Aliquots from column fractions (MES buffer) containing the 14-kD xylanase were concentrated 20-fold by means of Centricon-3 filter units (Amicon, Beverly, MA, USA); then, diluted 1:4 with sample buffer containing 50 mM dithiolthreitol and heated at 100°C for 2 min. Protein bands were detected by gel immersion in 0.1% Coomassie blue R-250 (Bio Rad Labs) in 40% (v/v) methanol/10% (v/v) acetic acid for 30 min. Stained gels were de-stained in the same solvent (methanol/acetic acid). Individual protein bands were excised manually with a razor blade and subjected to trypsin digestion. The resultant peptide library derived from 14-kD xylanase bands was analyzed by tandem mass spectrometry. Sequence information from this peptide library was compared with a data base of all predicted proteins annotated in the genome of *A. oryzae* (domesticated form of *A. flavus*) isolate RIB40 (<http://www.cadre-genomes.org.uk/Aspergillus-oryzae/>). The identity of the resultant protein product was confirmed by BLAST searches [14] against the annotations of *A. flavus* isolate NRRL 3357 (<http://www.aspergillusflavus.org/genomics/>) and against the NCBI protein data base (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Results

As anticipated from prior work with other Aspergilli, growth of *A. flavus* K-49 on larch xylan as a sole

carbon source resulted in expression of xylanase activity, which was completely absent in cultures grown on a sugar-rich medium (data not shown). This *A. flavus* xylanase was effectively inhibited by mercuric ion, although fairly high concentrations were required (Fig. 1). The curve generated by this inhibition data suggested a biphasic effect, with a sharp change between 0 and 10 mM. The ED₅₀ of Hg⁺² inhibition was 17.5 mM, with a maximum of 83% inhibition occurring at 50 mM.

Gel filtration chromatography of the K49 xylanase preparation in the P-30 medium afforded the separation of a small protein peak from the majority of other fungal secreted proteins. This peak (fractions 30–40) coincided with the major peak of xylanase activity (Fig. 2) and was selected for further analysis. A smaller activity peak was eluted in earlier fractions that contained multiple proteins. In addition, two major peaks of A_{280nm}-absorbing material eluted after the included volume of the column. One of these later-eluting A_{280nm}-absorbing peaks also demonstrated a small increase in conductivity (data not shown). These later-eluting A_{280nm} peaks appeared to correspond to yellow-brown and red-brown colored bands of sample material eluted from the column.

Analysis of fractions from the P-30 column peak corresponding to the major xylanase activity peak by denaturing gel PAGE revealed a major and minor band in the 14-kD region of the gel (Fig. 3). In addition, these fractions also revealed a very minor band at about 6 kD. It is possible that this small

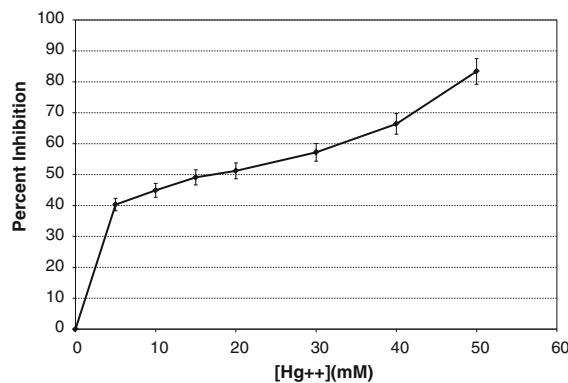


Fig. 1 Effect of mercuric chloride on *A. flavus* xylanase activity. Hg⁺² concentrations are expressed as mM. The standard radial diffusion xylanase assay was used to determine inhibition levels

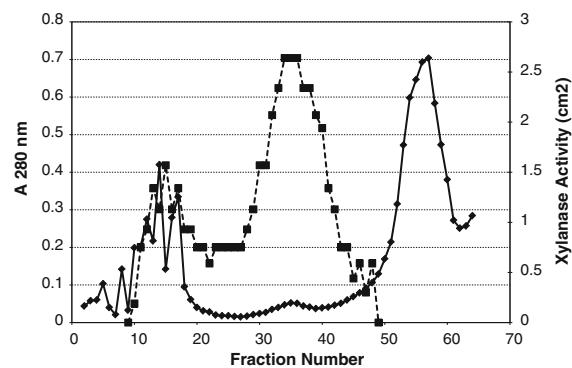


Fig. 2 Elution profile of *A. flavus* K49 xylanase preparation on a P-30 gel filtration column. Two-ml fractions were monitored for protein concentration (A_{280nm}) (solid line) and xylanase activity (dashed line) by the standard radial diffusion assay

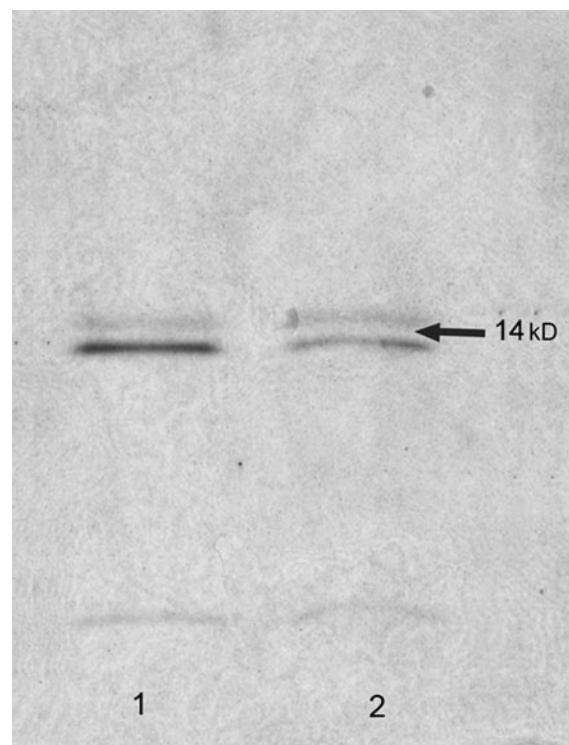


Fig. 3 Tricine-SDS PAGE analysis of P-30 column fractions containing small molecular weight xylanase activity. Samples in lanes 1 and 2 were derived from column fraction no. 34 and no. 35, respectively. The region of the gel where a 14-kD standard protein would migrate is indicated by the arrow. Proteins were visualized by silver staining

protein band was generated by the sample denaturation conditions (SDS, DTT, heat), since this small protein in the sample applied to the column should

have been resolved from larger proteins (e.g., 14 kD) by size exclusion. Thus, an effort was made to analyze these same fractions by native gel electrophoresis to avoid protein degradation in denaturation conditions. Previous work had revealed that proteins in these fractions (no. 34, 35) did not migrate in high pH (8.8) native gel conditions, suggesting the proteins of interest might be basic proteins. Thus, these fractions were subjected to analysis by acidic native PAGE. Acidic native gel analysis of these fractions yielded a single silver-staining band, suggesting a homogeneous protein preparation (Fig. 4). These fractions demonstrated unusual band morphology. Each lane with xylanase sample showed rounded “beads” of staining material on each side, connected by a thin band of staining material. This band morphology is reminiscent of glycoproteins [15].

Comparison of the 14 kD *A. flavus* xylanase peptide library to the *A. flavus/A. oryzae* genomic data base yielded a strong match to endo-1,4- β -xylanase (*XlnA*) of *A. oryzae* (canonical locus AO090120000026). A total of 89.2% of the derived spectra could be assigned to this genomic locus. In addition, the promoter sequence for this gene locus (*XlnA*) contains consensus binding sites for the following transcription factors: XlnR (5'-GGCTAA-3'), PacC (5'-GCCARG-3'), and CreA (5'-SYGGRG-3'). The remaining 10.8% of peptide fragments matched a single metalloprotease (AO0900010001 35).

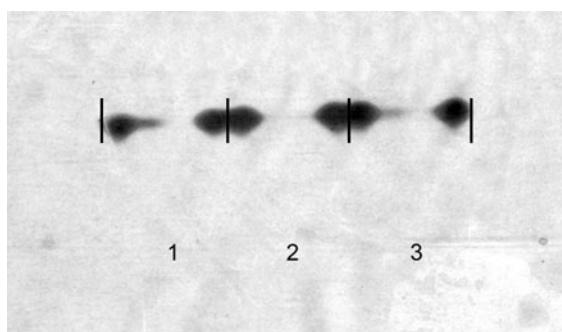


Fig. 4 Acidic native PAGE analysis of P-30 column fractions containing small molecular weight xylanase activity. Samples in lanes 1, 2, and 3 were derived from fraction nos. 34, 35, 36, respectively. The proteins migrated to the half way point in a 15% T gel. Proteins were visualized by silver staining. Note bead and strand band morphology

Discussion

Since plant xylans comprise a complex class of hemicelluloses, multiple hydrolase activities are required to catabolize these complex molecules. Endoxylanases break down the backbone consisting of β -1,4 linkages, with hydrolysis occurring at internal, unsubstituted residues on a random basis, yielding oligosaccharides. β -Xylosidases hydrolyze xylo-oligosaccharides to xylose. α -L-Arabinofuranosidases and arabinoxylan arabinofuranohydrolases remove arabinose residues. 4-O-Methylglucuronic acid residues are hydrolyzed by α -glucuronidases, while β -D-galactosidases remove D-galactose residues. Finally, acetyl and ferulic acid substituents are removed by specific esterases [16]. Fermentation of *A. flavus* in a medium containing larch (wood) xylan as the sole carbon source resulted in the secretion of multiple proteins (Fig. 2). In addition to endoxylanases, the probability is high that at least some of these other activities are expressed by the observed secreted fungal proteins.

Aspergillus xylanases have been shown to be sensitive to inhibition by some divalent transition metal ions. For example, the endoxylanases of *A. niger* are inhibited by 0.07 mM Hg⁺² [17], and *A. nidulans* β -xylosidase is inhibited by 1 mM Ag⁺², Cu⁺², and Hg⁺² [18]. Likewise, xylanase activity in *A. flavus* crude preparations is inhibited by mercuric ions, although at a higher concentration than seen in other Aspergilli (Fig. 1).

The observation of A_{280nm}-absorbing peaks of material eluting after the “included volume” of the column (where small non-interacting molecules would elute, e.g., NaCl) suggests these materials demonstrate additional interactions with the column matrix. That these materials are colored and demonstrate strong A_{280nm}-absorbing characteristics is consistent with limit xylans containing lignin moieties (present in medium carbon substrate) that show hydrophobic interactions with the acrylamide column matrix.

When crude *A. flavus* medium preparations were fractionated using P-30 gel filtration chromatography, a 14-kD peak was isolated with strong xylanolytic activity. Native acidic PAGE of this fraction revealed a single band, while SDS-PAGE showed two bands in the 14-kD range and a third in the 6-kD range. It is likely that the multiple bands observed in

SDS-PAGE are due to modification of the protein during sample denaturation. The minor 14-kD band may be due to the loss of small carbohydrate moieties; the 6-kD band may be due to protein degradation. Analysis of the 14-kD fraction by trypsin digestion/tandem mass spectrometry revealed peptide fragments from a single xylanase (*xlnA*; AO090120000026) of the five xylanases annotated in the *A. flavus* genome.

Examination of the genomic sequence of *xlnA* revealed the presence of consensus binding sites for the regulatory proteins XlnR, CreA, and PacC. In *A. niger*, XlnR induces the production of endoxylanases and β -xylosidase, along with a number of accessory enzymes important for hemicellulose digestion [19]. A homolog to the *A. niger* XlnR is seen in *A. oryzae*; both transcription factors have zinc binuclear cluster domains near the N-terminus, and the two proteins illustrate 78% sequence identity overall [20]. This raises the strong possibility that xylanases are regulated in a similar manner in the two Aspergilli.

In *Aspergillus*, CreA is responsible for carbon catabolite repression, in which target enzymes are not expressed in the presence of easily metabolized carbon sources (e.g., glucose, fructose, etc.). Endoxylanases and β -xylosidases have been shown to be repressed by CreA in *A. nidulans* [18, 21]. When control cultures of *A. flavus* were grown on a medium containing glucose as a sole carbon source, no xylanase activity was detected, suggesting that this enzyme is subject to carbon catabolite repression.

PacC is the global pH regulator in *A. nidulans*. It promotes transcription of alkaline-expressed genes (pH > 7) and represses transcription of acid-expressed genes [22]. PacC binding sites have been located upstream of *xlnA* and *xlnB* in *A. nidulans*. In the presence of D-xylose, *xlnA* was expressed in alkaline conditions and *xlnB* was expressed in acid conditions [23]. There is a PacC binding site upstream of the gene encoding the major endoxylanase (*xynF*) of *A. oryzae*, suggesting a role for pH regulation in xylanolytic enzymes.

We have identified a major source of xylanolytic activity when *A. flavus* is grown in a mildly acidic environment with xylan as a primary carbon source. Further study may elucidate both the genetic regulation of xylanases and the biochemical contribution of individual enzymes to the *A. flavus* necrotrophic mode of nutrient capture.

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